Regional Review

Diarrheagenic *Escherichia coli* in sub-Saharan Africa: status, uncertainties and necessities

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Abstract

Diarrhea is a leading cause of illness and death, particularly in developing countries. Enteropathogenic *Escherichia coli*, enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli*, enterohemorrhaghic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) have been associated with diarrheal disease in different parts of Africa, particularly among young children, HIV-positive individuals, and visitors from abroad. Each of these *E. coli* pathotypes uses distinct mechanisms, which are only partially understood, to colonize infected hosts and produce diarrhea. All known diarrheagenic *E. coli* pathotypes have been reported from diverse locations within Africa but the true burden from these pathogens is unknown because very few studies seeking these organisms with discriminatory methodology have been performed. Recent reports implicate ETEC and EAEC in a considerable proportion of childhood and travellers' diarrheas and suggest that some sub-types of these categories may have greater epidemiological significance than others. The significant contribution of EHEC to bloody diarrhea and hemolytic uremic syndrome is underappreciated because diagnostic capacity for this pathotype is generally inferior to that for confounders such as *Shigella* and *Entamoeba*. Recent studies in Africa have revealed the worrisome emergence of antimicrobial resistance and high asymptomatic carriage rates for diarrheagenic *E. coli* but bacterial and host factors that predispose to disease, as well as non-human reservoirs, are largely unknown. Future diarrheal disease research needs to focus on broadening the repertoire of pathogens sought in epidemiological surveys to include multiple categories of diarrheagenic *E. coli* while building capacity to detect these pathogens in local reference laboratories.

Key words: diarrhea; diarrheagenic *E. coli*; *Escherichia coli*; enteropathogenic *E. Coli*; enterotoxigenic *E. coli*; enteroinvasive *E. coli*; enteroaggregative *E. coli*; diffusely adherent *E. coli*; cell-detaching *E. coli*; antimicrobial resistance; Africa

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Introduction

Diarrhea is a predominant cause of childhood illness and death in developing countries. Even though tried-and-tested interventions mean that almost all diarrheal deaths are potentially preventable, Bryce *et al*. [1] estimated that between 2000 and 2003, sixteen percent of deaths of children under five in Africa were due to diarrhea. In recent years, childhood deaths from diarrhea have begun to fall worldwide, largely due to the introduction and widespread implementation of oral rehydration therapy. But in spite of heightened appreciation for risk factors such as unclean water, premature weaning, bottle-feeding, and malnutrition, the number of bouts of infantile diarrhea has risen steadily in the last decade and the decline in diarrheal disease deaths is least perceptible in the African continent [2-4]. Some children in developing countries experience over a dozen episodes of diarrhea in their first year. Repeated and persistent diarrhea in young children contributes to significant cognitive and growth impairment that can impact school performance and development [5-7]. Diarrhea makes a greater contribution to malnutrition and growth impairment than other common infections and *Escherichia coli* diarrheas may be even more detrimental than rotavirus infections in this regard [5,8,9]. The diverse range of diarrheagenic *E. coli* pathogenic types (pathotypes) and antigens means that children may be subject to repeated infection by different subtypes without immune protection.

Adults from lower burden countries commonly contract infectious diarrhea when they visit high-burden areas. In the late 1970s, the median attack rate among American Peace Corpers visiting Africa for six weeks or less was 54%. (This is probably an overestimate of the true traveller's diarrhea attack rate since Peace Corpers, in their early 20s, have typically had little previous travel experience and are more likely to engage in risky or adventurous behavior) [10]. In many cases, etiologic agents of travellers' and infantile diarrhea are similar but in

some cases they are not and the extent to which the etiology of these two syndromes overlap is not precisely known [3,11,12].

Diarrhea affects visitors to and children in hyperendemic areas most markedly but resident adults are also at risk of infection. Available data suggests that pathogens that typically cause persistent or invasive infections in children are often isolated from adults [13-15]. It is not clear whether this arises from bias since adults in endemic areas are least likely to seek care for diarrheal disease and, in Africa, these studies have always been performed at health institutions. Elderly patients may be as vulnerable to infection as young children and some pathogens can cause infections in individuals of all ages. More recently, HIV-infected patients have emerged as another hyper-susceptible sub-population from whom diarrheagenic *E. coli* pathotypes are often recovered [16-18].

Infectious diarrhea can be caused by viruses and bacteria as well as parasites, and patients may have mixed infections. Few studies look for major pathogens in all classes and even fewer studies seek supposedly minor pathogens so that the true contribution of many agents to disease is uncertain. Biomedical and clinical research has focused on those agents that are mostly likely to cause life-threatening illness, to spread rapidly, or to be vaccine controllable. Epidemiological research is also biased towards those agents that are most easily detected. Rotavirus, *Salmonella* spp. and *Shigella* are among the most investigated etiologic agents, particularly in Africa, for many of the aforementioned reasons, and diarrheagenic *E. coli*, which are difficult to differentiate from commensals, are less frequently sought.

In studies that have sought a broad range of pathogens, diarrheagenic *E. coli* have repeatedly featured as predominant causes of diarrhea. Brooks *et al*. [19] recently identified diarrheagenic *E. coli* in 20% of specimens in which a bacterial pathogen was detected; 34% of those were from children less than 5 years old. In Mozambique, diarrheagenic *E. coli* were recovered from 41.8% of children with diarrhea and 18.2% of controls. In the same study, rotavirus was detected in stools from 18.2% and 5%, and parasites from 37.8% and 56.8% of children with diarrhea and controls respectively [20]. A Tanzanian study found that screening for diarrheagenic *E. coli* increased the proportion of cases for which a pathogen could be identified by 34.6% in the dry season and 28% the rainy season to over 70% overall [21]. Conversely, studies that have failed to seek more than one class of diarrheagenic *E. coli* have often reported low rates of pathogen recovery [22].

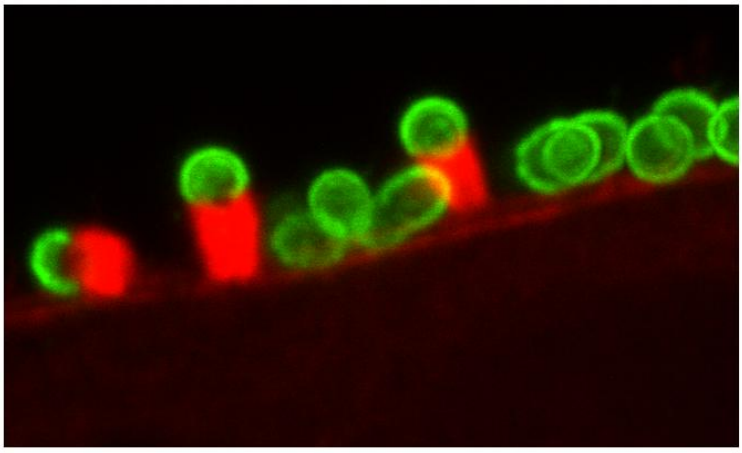
Diarrheagenic *E. coli* have been recognized as intestinal pathogens since the 1940s when Bray hypothesized that that *E. coli* subtypes might account for common infantile diarrhea of unknown etiology and identified a strain of *Bacterium coli* var neapolitanum, or enteropathogenic *E. coli* O111, as a probable cause of infantile diarrhea [23]. Using the Kauffman scheme of O:H serotyping, Neter was able to conclusively test the Bray hypothesis and report that certain "enteropathogenic" serovarieties of *E. coli* showed close association with infantile diarrhea [24]. Their etiologic role in diarrhea was verified by Levine *et al*. in volunteer challenge experiments reported in 1978 [25]. Half a century of microbiology research revealed that most "enteropathogenic" serotypes harbored virulence genes that are absent in non-pathogens so that by 1998, five categories of diarrheagenic *E. coli* that had been unequivocally associated with diarrhea were known and at least three more categories were under evaluation [26].

There are geographic variations in the epidemiology of different pathotypes and their sub-types, and surveillance for diarrheagenic *E. coli* remains weak. Although some pathotypes show promise for vaccine development, not enough is known about predominant subtypes to assure that vaccines will be effective in the places where they are most needed. Many of these problems are global but each is accentuated in sub-Saharan Africa due to a combination of a high burden from diarrheal infections and diagnostic insufficiency [27].

Enteropathogenic E. coli

The locus for enterocyte effacement (LEE) is a chromosomal pathogenicity island that confers a distinctive "attaching and effacing" phenotype (Figure 1). Enteropathogenic *E. coli* (EPEC) are diarrheagenic *E. coli* that have the LEE but do not carry genes for the phage-borne Shiga-toxins of enterohemorrhagic *E. coli*. Typical EPEC strains also carry a virulence plasmid, which bears genes encoding bundle-forming pili, the plasmid encoded regulator and other putative virulence genes. Evolutionary research has identified four major

Figure 1. Attaching and effacing by an enteropathogenic *E. coli* strain intimately adhered to cultured human epithelial cells.



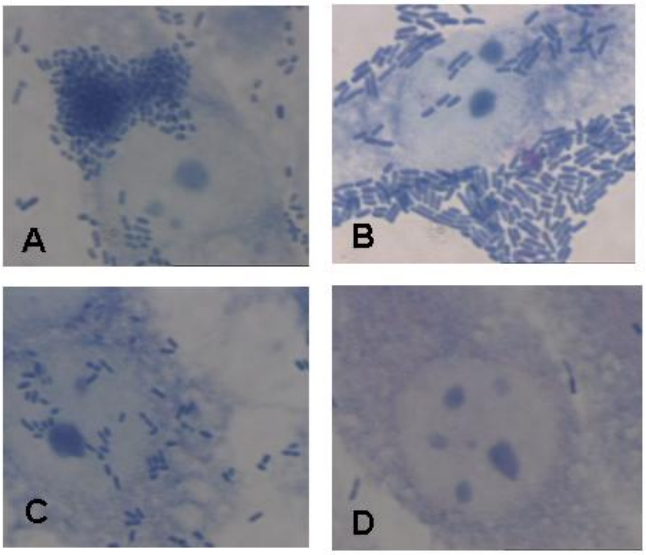
lineages of typical EPEC, suggesting that this pathotype emerged by horizontal acquisition of key virulence loci multiple times [28,29].

Cravioto [30] and Tzipori *et al*. [31] must be credited for devising the first serotype-independent means for delineating EPEC. By identifying localized adherence and attaching-and-effacing as phenotypes associated with EPEC pathogenesis, they also paved the way for molecular studies that led to the discovery of major EPEC virulence factors. Within two decades of their reports, the localized adherence phenotype (Figure 2a) was attributed to bundle-forming pili (BFP) encoded on the EPEC-adherence factor (EAF) plasmid, and the attaching-and-effacing phenotype to the chromosomal LEE pathogenicity island. The current model of typical EPEC infection begins with BFP-mediated localized adherence. Bacteria then deliver a translocated intimin receptor (Tir) to infected host cells by means of a LEE-encoded type III secretion system. The Tir protein inserts into the host membrane, providing a receptor for the bacterial outer-membrane protein, intimin, also LEE encoded. Intimin-bound Tir is tyrosine phospohorylated in the host, beginning a cascade of signaling events that ultimately lead to reorganization of the host cytoskeleton around the bacteria [32] (Figure 1). A number of effector proteins, encoded within and outside of the LEE, are delivered via the type III secretion system and likely contribute to diarrheagenicity [33]. Additionally, EspC, an autotransported enterotoxin which also confers lysozyme resistance, is secreted by some strains [34,35].

In the 1970s and 1980s, "classical" EPEC serotypes were associated with disease in many parts of Africa, suggesting that EPEC was a predominant cause of diarrhea at that time. These data should be interpreted with caution, in part because they were based on O:H-serotyping, or O-serogrouping, alone but also because very few studies performed in Africa were controlled before 1985. Notwithstanding, in South Africa, one study identified Rotavirus and EPEC as the most common cause of childhood diarrhea and throughout the 1970s and 80s, summer diarrhea had similar prevalence and etiology as did this syndrome in the Northern hemisphere until the 1960s [36-38] and in South America to this day. East, Central and West African studies using serological methods found EPEC highly prevalent [39-44].

Although O- or O:H-typing was the standard diagnostic test for EPEC until the 1980s, many early African studies do not record the O-groups detected, potentially because they employed pooled antisera [41]. However, when specific O-groups were reported, EPEC O111 was often predominant and documented in outbreaks with high fatality rates in Africa [39,42,44-48]. O111 EPEC were similarly recovered from nursery outbreaks in the US from the early and mid-1900s and EPEC strain B171-8, a prototypical strain from a US nursery outbreak, possesses the LEE, BFP and other virulence-plasmid borne genes as well as other loci hypothesized to contribute to disease [49,50]. Examination of isolates from a Kenyan outbreak suggests that the etiologic O111 strain was a typical EPEC isolate [47] but in-

Figure 2. Adherence patterns of diarrheagenic *E. coli* to cultured epithelial cell A. Localized Adherence by enteropathogenic *E. coli*, B. Aggregative Adherence, C. Diffuse Adherence D. Non-adherent control strain.



depth molecular characterization of O111 isolates is yet to be performed. A large partially conserved resistance plasmid has recently been described in O111 EPEC and other EPEC2-lineage strains [51] but isolates from Africa, which are often multiply resistant [47], have not been screened for this element.

When data from controlled studies in Africa became available, the significance of EPEC as a pathogen in published literature began to decline [20,52-56]. Due to a number of beneficial advances, it is impossible to precisely pinpoint a reason(s) for this decline. Breastfeeding is protective against diarrhea, and against EPEC in particular. It is possible that that EPEC became a less predominant cause of diarrhea as a consequence of the UNICEF/WHO 0-6 month exclusive breastfeeding campaign [57-59]. At the same time, the discovery of BFP and the LEE [60,61] permitted researchers to use molecular methods and/or adherence assays for EPEC identification. As some, but not all, EPEC so identified belong to classical EPEC serogroups, and there may be non-EPEC strains that express EPEC O:H antigens, there is only partial overlap between EPEC as defined after 1990 and EPEC reported in earlier studies. This is illustrated by a 1990 report from Djibouti, which identified 28 "EPEC" belonging to classical serogroups, but only ten of these showed localized adherence, even though typical EPEC show this phenotype [62]. Thirdly, earlier data is predominantly from South Africa and/or urban areas. Data from other parts of Africa and from provincial and rural regions where the etiology of diarrheal disease could differ considerably has been more commonplace in recent years [19,56,63-65]. Thus it is unclear whether EPEC infection has declined due to interventions, or whether earlier studies produced an overestimate of the relative contribution of these organisms to the overall diarrheal disease burden.

Having documented these uncertainties, it is important to emphasize that EPEC has been, and remains, an important cause of diarrheal disease, even if it may be subordinate to some other diarrheal pathogens in this role [66]. EPEC infections are most common, and most serious, in children under one year of age, and continue to be associated with disease in this age group, as illustrated by recent reports from Mozambique and Botswana [56,67]. Additionally, EPEC has emerged as an important cause of diarrhea in HIV-infected individuals. A Central African Republic study found that EPEC from classical serogroups, that also showed localized adherence and hybridized to the *eae* probe, were strongly associated with chronic diarrhea in these patients ($p < 0.001$)[68].

With regard to virulence factor content, two major subtypes of EPEC are presently recognized. Typical strains harbor the LEE and the EAF plasmid while atypical strains have the LEE but lack the EAF-plasmid or EAF-associated virulence plasmid. The definition is fuzzy in that some "atypical" strains are unavoidably typical isolates that lost the EAF after isolation and others contain parts of the plasmid but do not express bundle-forming pili [69,70].

However, available data appear to suggest that typical strains belong to a few well-defined clones, which were represented by the earlier-defined "classical" serotypes, including those implicated in well-investigated nursery outbreaks, whereas there are many more lineages of atypical EPEC [71,72]. More recent evidence has emerged to suggest that atypical EPEC may play a more important role in persistent diarrhea [73].

Few African studies have attempted to delineate typical and atypical EPEC but examples of both that do not belong to classical serovars exist and have been recovered in Africa. Vila *et al*. identified 21 EPEC isolates from 346 diarrheal stool specimens in Tanzania [64]. They were able to identify *eae*, a gene on the LEE, in 20 of these strains but the plasmid loci *bfp* and/or EAF were found in only eight. Similarly, in Mozambique, nine of 16 EPEC strains identified lacked *bfp* and showed localized as opposed to diffuse adherence [20]. Thus typical as well as atypical EPEC are common and diagnostic tests that delineate EPEC lineages rather than detect specific virulence factors are poorly predictive of EPEC in much of Africa where epidemiology has not been well defined. Lacher *et al*. [28] have shown that EPEC isolates from a single study performed in Guinea Bissau represent diverse lineages, emphasizing the need to employ virulence gene or virulence phenotype-based detection systems. Although popular and requiring no extraneous facilities for use, EPEC O-antisera is not a cost-effective tool for identifying this pathogen. PCR or hybridization for LEE genes such as the *eae* gene, encoding intimin, virulence plasmid loci such as *bfp*, or the demonstration of localized adherence or the attaching-effacing phenotype represent more reliable ways to identify EPEC. Unfortunately, as molecular and tissue-culture facilities are not widely available, many diagnostic laboratories and epidemiological researchers continue to use O-group typing as the sole means for identifying EPEC in many places [74,75].

Enterotoxigenic E. coli

Upon realization that some *E. coli* isolates from patients with diarrhea could induce fluid accumulation in a ligated rabbit ileal loop model, it was found that some enterotoxigenic *E. coli* (ETEC) elaborated toxin very similar to cholera-toxin [76,77]. In the 1970s and early 1980s, strains producing this heat labile enterotoxin and/or a heat stable enterotoxin were among the most common causes of diarrhea in travellers visiting Kenya [78,79]. Those studies also revealed that that ETEC isolates from Kenya were commonly antimicrobial sensitive, and that antimicrobials could be administered to prevent traveller's diarrhea. Similar reports implicated ETEC in infantile diarrheas in Kivu Province, Zaire, and Lagos, Nigeria [52,80,81]. Early studies identified ETEC by screening for toxigenic activity of isolates or with immunologic reagents that agglutinated the heat-stable or heat-labile enterotoxin. The transition from phenotypic to genotypic definition of ETEC was less confounding than with EPEC because both tests essentially measured the same thing—enterotoxigenicity [41,82,83], which makes older studies comparable to modern ones.

ETEC is one of the best documented and predominant causes of diarrhea in travellers visiting African countries from Europe and North America [12,84-86]. ETEC vaccines could therefore be revenue-generating and their development is consequently an area of priority research [11,87]. ETEC is also a major etiologic agent of infantile diarrhea in African countries, which could benefit considerably from vaccine development [66,88]. Among children less than five years of age, ETEC is strongly associated with disease and in many studies was the most common cause of acute diarrhea [65,66,80,89]. ETEC can quickly produce potentially fatal dehydration in young children and has been associated with life-threatening persistent diarrhea in those who are malnourished [86,90]. Although less common, ETEC can also cause diarrhea in older residents in endemic areas [86]. One study in rural Tanzania found that ETEC diarrheas were significantly more likely to occur in the dry than in the rainy season [21]; however, it is possible that different ETEC subtypes vary in their seasonality [86].

ETEC strains colonize the small intestine and typically cause a watery diarrhea. Abdominal pain and vomiting are less common than with many other enteric pathogens [91]. ETEC strains elaborate a heat labile toxin (LT), which is structurally and mechanistically similar to the more active cholera toxin, and/or a heat stable toxin (ST). There are two types of ST – the human (STh) and "porcine" (STp) varieties, although the latter is also implicated in human disease. While there are also two types of LT, only LT-1 is believed to play a role in human disease [26]. LT, which structurally and functionally resembles cholera toxin, was initially thought to be the more significant than ST. However, a large US

nursery outbreak in 1974-75 was attributed to ST-producing ETEC [92] and shortly after, Levine *et al*. [93] demonstrated that strains producing ST alone were capable of eliciting diarrhea, nausea, vomiting and abdominal cramps in adult volunteers. This finding is backed by epidemiological evidence, including controlled studies performed in different parts of Africa, which suggest that ST-producing ETEC are more strongly associated with childhood diarrhea than are LT-producing strains, even though the latter may be more common overall [41,65,91,94]. The same may also be true for travellers' diarrhea [95]. The greater significance of ST is of direct consequence to vaccine development because this small toxin is only immunogenic when conjugated: LT vaccines are much easier to design.

In theory, ETEC adhesins, so called coli-surface (CS) antigens, represent a more useful target for vaccine development than toxins; however, there are over 20 known CS antigens and while a multi-component vaccine is a possibility, the relative importance of each one varies with geography [26,86,96,97]. Some CS antigens assemble into fimbrial or fibrillar structures called colonization factor antigens (CFAs) of which there are four morphological types. Longus, a bundle-forming, pilus, is comprised of CS21 units and appears to be more common in ST-producing strains [98,99]. Between 8% and 36% of ETEC isolates from different locations express the longus pilus, generally in conjunction with other CS antigens [100-103]. ETEC strains may possesses one or more non-fimbrial adhesins (recently reviewed by Fleckenstein *et al*. [104]). One of these, EtpA, is a glycoprotein that links flagella to host cells and may be a potential vaccine candidate [105,106].

A Kenyan study focusing on travellers suggested a predominance of CFA/IV and similar findings were made in an earlier study that examined ETEC isolates from Zaire and Rwanda [95,107]. CFA/IV is typically comprised of CS6 with CS5 or CS4. Steinsland *et al*. [94] sought to determine the role of ETEC in infantile diarrhea and to identify predominant CFAs in Guinea-Bissau. They performed a longitudinal cohort study in which they collected weekly specimens from 200 children aged over one to two years and identified 1,018 distinct ETEC infections over the study period. More than one ETEC strain was recovered from as many as 89 infections and sporadic as well as epidemic infections were detected. Furthermore, 565 ETEC isolates carried the LT-encoding genes alone and the remainder were roughly equally divided between ST alone-positive strains and strains possessing genes for both toxins [94]. Odds ratios for diarrhea only exceeded 2.0 in infections caused by strains bearing the STh toxin, with apparent disregard to CS antigen, or LT-positive strains with select colonization factors, notably CS7. STh was more commonly associated with epidemics. With respect to colonization factors, CS6 was the most common CS antigen, alone or in combination with other antigens, but the CS6-CS5 combination, which was predicted to be important in travellers' diarrhea studies from Kenya and central Africa [95,107], was seen in only 26 (2.6%) isolates from the Guinea Bissau study. CS17, prominent in central African isolates from over a decade before [107], was found in only 21 LT-positive isolates and CS21, the longus pilus surface antigen, was seen in 11 isolates, all of which were ST-positive.

Interestingly, Steinsland *et al*. [94] recovered ETEC strains bearing almost all known types of colonizing factor antigens and many strains were negative for all known CS antigens. CS13, CS15, CS18, or CS19, considered uncommon due to reports from other areas, were common among ETEC from Guinea-Bissau, supporting to the idea that knowledge about so-called common factors should not bias studies in previously uninvestigated parts of the world. The value of this large and in-depth study is considerable. Further analysis revealed that previous ETEC infection was protective in Guinea Bissau children, but that although LT conferred protection against subsequent LT-ETEC infection, surprisingly, CS antigens did not confer cognate protection [108]. Even though adhesins are generally good vaccine candidates, this may not be so for ETEC.

There are varieties of ETEC that cause diarrhea in livestock, including pigs and calves. Almost a third of porcine *E. coli* examined in Zimbabwe were positive for one of the LT or ST variants or a Shiga-toxin gene [109]. For most ETEC, host selectivity is defined by surface antigen tropism and most animal isolates have other adhesins [110] but least some animal ETEC may also have the potential to cause disease in humans [111]. One study appeared to suggest that person-to-person transmission of ETEC was unlikely, at least among healthy adults in America (likely due to high infectious dose) but similar studies have not been performed with children or in Africa [112]. ETEC are believed to be primarily transmitted via contaminated food and water. ETEC strains have been recovered from river water in

Kenya but other studies tracking ETEC in non-human specimens are needed [113].

Antimicrobial resistance of ETEC is a growing concern. The toxin genes in these bacteria are borne on plasmids that may also bear antimicrobial resistance genes and in some cases are self-transmissible [114-117]. ETEC strains could additionally acquire resistance on mobile elements distinct from the toxin-bearing plasmid. In both scenarios, antimicrobial use provides selective pressure for toxigenic strains and plasmids. Resistance rates to previously effective drugs such as the tetracyclines, trimethoprim-suphamethoxazole and ampicillin have risen to 30-90% in some parts [62,64,85,95,117]. As a consequence, quinolones became the drugs of choice for traveller's diarrhea but resistance to these drugs has also emerged. As with other *E. coli*, this is typically due to point mutations in the quinolone resistance determining regions of the *gyrA* and *parC* genes although other, more recently described, mechanisms are yet to be investigated [118,119].

Enteroinvasive E. coli *and* Shigella

Shigella spp are the etiologic agents of bacillary dysentery and a common cause of travellers'[85], childhood, and adult diarrheal disease. Dysentery is a typically non-voluminous, invasive diarrheal syndrome that may, but not always, be accompanied by gross blood, leukocytes and/or mucus in stool. The interested reader is referred to extensive *Shigella*-specific reviews [120-122]. Unlike other *E. coli*, *Shigella* rarely ferment lactose at all, ferment other sugars aerogenically, decarboxylate lysine, and are non-motile in conventional laboratory media. For this reason, *Shigella* was earlier assigned a separate genus but molecular data has revealed that *Shigella* are in fact *E. coli* species [123]. Because *Shigellae* cause a well-defined disease syndrome, the façade of a separate genus has been maintained in clinical microbiology in spite of its acknowledged biological inconsistency. Enteroinvasive *E. coli* (EIEC) classify biochemically as *E. coli* but share many properties, including virulence mechanisms, with *Shigella*. The shared properties of different *Shigella* and EIEC arise from evolutionary convergence rather than a recent shared common ancestor [124-126].

Shigella and EIEC possess a large invasion plasmid, which encodes the Mxi-Spa type III secretion system and invasion plasmid antigen (Ipa) effectors, conferring upon these bacteria the ability to invade eukaryotic cells. The plasmid also encodes IcsA, which makes it possible for bacteria to travel from cell to cell *in vivo*, eluding the immune system. *Shigella dysenteriae* type I has Shiga toxin genes and is associated with epidemic dysentery. Other *Shigella* and EIEC may carry genes encoding one or more *Shigella* enterotoxins. They typically lack many *E. coli* metabolic genes and the loss of some of these loci is pathoadaptive [127,128].

It is important to understand local *Shigella* epidemiology and susceptibility patterns because the infectious dose of these organisms is low and antimicrobials, which are indicated in bacillary dysentery, must often be prescribed empirically. Fortunately, *Shigella* is among the best studied diarrheal pathogens in Africa, largely because it can be isolated, identified and even subtyped by aerobic cultural methods and serotyping alone. However, generation and dissemination of epidemiological data still falls short of need. *Shigella flexneri* is the serovariety that predominates in developing countries, including those in Africa, although virtually all serovars are recovered in medium to large studies [129-132]. *Shigella* remains an important etiologic agent of diarrheal disease in older children and adults [19]. It is probable that EIEC shares this epidemiological feature [133] but it is rarely sought.

EIEC often ferment lactose, making them almost as difficult to delineate from commensals as other diarrheagenic *E. coli*, and consequently their epidemiology in Africa is much less well studied than *Shigella*. EIEC can most easily be detected by probing for the invasion plasmid, which is also present in *Shigella*. EIEC can also be distinguished from other *E. coli* by measuring invasion in tissue culture cells or guinea-pig conjunctivae (the Sereny test). EIEC strains, like *Shigella* and unlike most other *E. coli*, will also often bind Congo red, ferment sugars anearogenically, fail to ferment lactose or to decarboxylate lysine. However, although biochemical tests are valuable pre-screens, none show sufficient sensitivity or specificity to be considered diagnostic for EIEC.

Over the last decade, EIEC were sought but not found in one Gabonese study and studies from Kenya, Mozambique, Ghana and Nigeria also identified a small number of EIEC isolates but saw no significant association with disease [14,15,20,134-136]. In Senegal, *ipaH*-positive *E. coli* (*ipaH* is a marker for *Shigella* and EIEC) were recovered from 16 of 279 people with diarrhea and 8 of 276 controls

Table 1: Characteristics of diarrheagenic *E. coli* pathotypes.

	Evidence for diarrheagenicity	**Adherence Pattern**	**Principal Virulence Factors**
Enteropathogenic *E. coli* (EPEC)	Epidemiologic associations, Outbreaks, Human volunteer challenge	Localized	Locus for enterocyte effacement (LEE) encoding intimate adhesin, intimin, type III secretion system and secreted effectors, secreted effectors encoded outside the LEE, bundle forming pili and other virulence-plasmid genes, EspC enterotoxin
Enterotoxigenic *E. coli* (ETEC)	Epidemiologic associations, Human volunteer challenge	Weak Diffuse	Heat stable toxin (ST), Heat labile toxin (ST), colonization factor antigens (CFAs), longus pilus, Tia invasin
Enteroinvasive *E. coli* (EIEC) and *Shigella*	Epidemiologic associations, Outbreaks	Variable	Invasion plasmid encoding type III secretion system and secreted effectors, IcsA, *Shigella* enterotoxins, Shiga toxins (Stx; *Sh. dysenteriae*), pathoadaptive deletions in house-keeping genes
Enterohemorrhagic *E. coli* (EHEC)	Epidemiologic associations, Outbreaks	Variable	LEE, Shiga toxins (Stx), virulence plasmid encoded enterohemolysin, accessory adhesins
Enteroaggregative *E. coli* (EAEC)	Epidemiologic associations, Outbreaks, Human volunteer challenge	Aggregative	Dispersin, Aggregative Adherence Fimbriae (AAF; multiple types), Plasmid-encoded toxin, EAEC heat-stable toxin (EAST-1), accessory adhesins
Diffusely-adherent *E. coli* (DAEC)	Epidemiologic associations	Diffuse	Diffuse adhesin (Daa), AIDA adhesin, other adhesins
Cell-detaching *E. coli*	Epidemiologic associations	Detaching	Alpha hemolysin, P-pili, Cytotoxic Necrotizing Factor
Cytolethal-distending toxin-producing *E. coli*	-	Variable	Cytolethal distending toxin

($P < .000001$) [137]. Other studies have invariably found proportionately more EIEC in cases than controls and the failure to detect disease association appears to be linked to overall low prevalence of these pathogens and the small sample size of these studies. EIEC may be at least as prevalent as *Shigella* in some areas but studies specifically designed to study EIEC may be needed to verify this [14,65].

Vargas *et al*. found that sporadic *Shigella* infections are more common in the dry than in the rainy season in Tanzania ($P = 0.02$) [21], a feature that agrees with the association of poor-quality water as the most significant risk factor for dystentery in the Limpopo Valley of southern Africa [138]. *Shigella* have a low infection dose, in part conferred by acid resistance that also makes survival in low-pH foods possible, and these bacteria have been isolated from a variety of street-vended foods in Addis Ababa, Ethiopia [139] and Accra, Ghana [140].

Survival at low pH is also of significance in parts of Africa where fermented corn gruels of various types are the principal weaning food and acid adapted *Shigella* can survive for up to two days [141]. EIEC and *Shigella* have not been reported as predominant causes of diarrhea in HIV patients. In north-west Ethiopia, *Shigella* was recovered from 3.5% of HIV-1-seropositive patients, 5.1% of HIV-1-negative patients with diarrhea, and 2.5% of healthy controls. *Shigella* showed no association with disease or with diarrhea in HIV-1-positive patients [142]. Similar findings were made in Senegal and the Central African Republic [143,144].

Sh. dystenteriae type I can cause large epidemics in people of all ages and may also show seasonality [145]. This highly virulent serotype produces Shiga toxin, which exacerbates bloody diarrhea and can also precipitate hemolytic uremic syndrome [146-149]. In the last decade, large *Sh. dysenteriae type* I outbreaks have been documented from the Central African Republic (2 outbreaks) [150], Cameroon [151], Sierria Leone [152], Zimbabwe [153], South Africa [154], and Kenya [155], in addition to numerous sporadic reports. Molecular studies suggest that a few *Sh. dysenteriae* clones account for epidemics over several decades worldwide and these clones' bacteria move through large geographical areas periodically flaring epidemics and then disappearing or relocating [156,157]. Many epidemics in Africa may in fact be connected and the

absence of reports from other areas is likely failure to detect, report or publish outbreaks.

Shigella isolates are increasingly multiply-resistant. Bogaerts *et al*. [158] recorded a progressive increase in resistance of *Shigella* isolates from Rwanda to ampicillin, chloramphenicol, tetracycline, trimethoprim and sulphonamides between 1983 and 1993, and similar reports come from Ghana [159]. Resistance is also highly prevalent among all *Shigella* serovars from Sudan, Kenya, Nigeria and Ethiopia [129,131,160,161]. A class 2 integron bearing cassette encoding resistance to trimethoprim, streptomycin, and spectinomycin has been described in *Sh. sonnei* from Senegal [162]. Recent studies [158] have additionally observed the emergence of nalidixic acid resistance and subsequent rapid increase in the prevalence of resistance to that drug upon its introduction. Widespread resistance prompts concern antimicrobials are indicated in invasive and potentially life-threatening *Shigella* infections. A combination of diagnostic insufficiency, inadequate surveillance, and limited drug choice means that many patients in Africa infected with *Shigella* do not receive an antimicrobial to which the infecting organism is susceptible [163]. *Shigella* outbreaks have been associated with complex emergencies following civil conflict and case fatality rates are high when etiologic organisms are multiply-resistant [152,164].

Enterohemorrhagic E. coli

Reports of hemorrhagic colitis- and hemolytic uremic syndrome-associated *E. coli* first appeared in the literature in 1983, when Karmali *et al*. demonstrated their cytotoxicity [165,166]. In the same year, O'Brien *et al*. reported that *E. coli* O157:H7 isolates from cases of hemolytic uremic syndrome produced a toxin later shown to be phage-borne and identical to the *Sh. dysenteriae* type I Shiga toxin (Stx) [166,167]. In addition to Stx, enterohemorrhagic *E. coli* (EHEC) strains additionally contain the LEE, a plasmid-borne enterohemolysin and other virulence genes (Table 1). Stx-positive *E. coli* that lack the LEE and enterohemolysin are referred to as Shiga-toxin producing *E. coli* (STEC) and at least some STEC are also highly virulent pathogens. STEC have been reported from a number of locations, including Nigeria [65], Uganda [168] and the Central African Republic [68]. In the Central African Republic, they have been associated with hemorrhagic colitis and hemolytic uremic syndrome in HIV-positive patients [68].

Because EHEC was initially associated with bulk-produced beef products in North America and Western Europe, it was earlier thought that these pathogens would not be seen in developing countries. The first documentation of EHEC in Africa was a sporadic case of hemorrhagic uremic syndrome caused by EHEC O157:H7 and reported from South Africa in 1990 [169]. Three years later, a South African laboratory described one of the largest EHEC outbreaks in the world, which began on a sugar plantation in Swaziland, and resulted in approximately 2,000 deaths [170,171]. The spread of the organism is likely to have been augmented by contamination of surface water by dead and dying cattle, or consumption of meat from there, which was in turn exacerbated by a drought [171].

In 1996, Germanii *et al*. [172] described an EHEC outbreak in Central African Republic. Like subsequent EHEC epidemics in Africa, it was mistaken for a *Shigella* outbreak and it was only molecular tests performed abroad that revealed that EHEC was the culprit. In 1997, a bloody diarrhea outbreak involved altogether about 300 cases—about a quarter of the residents of small town Ngoila in Cameroon. *Shigella* was suspected but subsequent molecular analysis at the laboratories of Centre Pasteur in Yaoundé and the Institut Pasteur of Bangui showed that the outbreak was of mixed etiology and that *Sh. dysenteriae* type 1, *Sh. boydii*, *Entamoeba histolytica* and enterohemorrhagic *E. coli* were present in stool specimens [151]. EHEC O157 was identified in 12 of 22 specimens examined in detail. It was impossible to determine which of the etiologic agents contributed most to the 16.4% mortality rate but the high number of fatalities was almost certainly linked to inadequate use of rehydration therapy and inappropriate antimicrobial use [173]. The report had important implications because Ngoila does not have easy access to a city with international connections. At the time of the outbreak, it was only accessible by motorbike or canoe [173], effectively refuting the supposition that EHEC was solely a western pathogen.

Even though multiple outbreaks had by that time been documented, a 1998 outbreak, this time due to O111 EHEC in Nigeria, was detected only because a case-control study for diarrheagenic *E. coli* was in progress. [14]. EHEC was significantly associated with diarrhea in that study (even with outbreak cases removed) and was the most common bacterial

pathogen recovered from bloody diarrhea specimens in which only one pathogen was identified [14]. Phenotypic tests commonly used to detect EHEC including sorbitol non-fermentation and agglutination of O157 antisera failed to identify most of the EHEC isolates in that study. In almost all EHEC outbreaks, the causative organism has been identified outside the country and after the outbreak has abated. Moreover, EHEC outbreaks have typically co-occurred with outbreaks of other diarrheal pathogens [14,167,171-173]. These factors suggest that many more epidemics occur in Africa than are reported and that diagnostic and surveillance capacity for EHEC is required.

WHO guidelines, which often reflect capabilities rather than necessities, highlight *Shigella* and *Entamoeba histolytica* sensu stricto as the predominant causes of dysentery in Africa. Most other recommendations concur [174]. In addition to outbreaks, sporadic EHEC isolates have almost always been found when sought in Africa [14,21,65,89,133,143,172,175-177]. Although the isolation rate was low in all these instances, they further emphasize the importance of considering EHEC in the differential diagnosis of bloody diarrhea. EHEC cause drastic clinical syndromes—hemorrhagic colitis, hemolytic uremic syndrome and thrombocytic thrombocytopenic puerpera, but these diagnoses are likely missed in many parts of Africa because of the still persistent belief that EHEC are not an important cause of disease locally and the endemnicity of *Shigella* that also produce these syndromes [149,171].

EHEC outbreaks in North America and Western Europe have been associated with undercooked beef, vegetables fertilized with cattle manure, and zoonotic or person-to-person transmission. Bovines have emerged as an important reservoir for EHEC in Africa as well [168,178]. In the Central African Republic, Germani *et al*. [172] associated EHEC infection with eating *Kanda,* a snack consisting of meat from a small bovine referred to as Zebu and marrow steamed in banana leaves. Steamed *Kanda* is sold days after preparation at roadsides, at ambient temperatures [172]. Recovery rates from Zebu animals were in the range of 28% [178]. Majalija *et al*. recently demonstrated that indistinguishable EHEC strains could be recovered from cattle and infected children from semi-nomadic pastoralist communities in South Western Uganda [168].

Multiple studies have shown that EHEC, with *Shigella* and *Entamoeba,* is an important cause of bloody diarrhea in Africa even though there is little or no diagnostic capacity to detect these organisms [14,179]. As recommended treatments for these pathogens differ considerably, there is need to increase diagnostic capabilities and determine relative culpabilities for all three. Many EHEC O157 strains are unable to ferment sorbitol or to produce β-glucuronidase and are not inhibited by tellurite. These properties have been exploited to develop selective-diagnostic media for *E. coli* O157. However, these media do not produce characteristic patterns with all O157 strains and fail to identify non-O157 EHEC [180,181]. Unfortunately, many recent studies have focused solely on EHEC belonging to this serotype [182,183]. A small study comparing diarrheagenic isolates from Kenya with strains from Japan observed a much broader variety of serotypes in the former [134]. One of the most thorough searches for EHEC and STEC—in bovines and humans—recovered strains belonging to a range of serotypes but none of these were O157 strains [168]. That study demonstrates that molecular tests, or possibly toxin-detection tests, offer the most promise for detecting these pathogens in Africa.

Enteroaggregative E. coli

In 1987, Nataro *et al*. [184] observed that while EPEC adherence factor (EAF) plasmid-positive strains belonging to classic EPEC serogroups invariably showed localized adherence to HEp-2 cells in culture, some other diarrhea-associated *E. coli* strains showed either diffuse adherence or a stacked-brick pattern that they termed aggregative (Figure 2). Localized- and aggregative- adherent *E. coli* were significantly more often isolated from children with diarrhea than controls. This finding was borne out by subsequent epidemiology studies which observed a strong association of EAEC with persistent diarrhea in India and Brazil [184-186]. Although only defined in 1987, with a role in disease that remained equivocal over the next decade, EAEC is now known to be a predominant cause of diarrheal disease in developed as well as developing countries [20,187-190]. EAEC were originally associated with persistent diarrhea but are now known to be associated with a wide range of diarrheal syndromes, ranging from watery to invasive diarrhea, which may be acute or persistent [191-193]. EAEC may be as important a cause of travellers' diarrhea as ETEC [193,194]. However, strain heterogeneity and an insufficient understanding of this pathotype preclude vaccine development at present, even though a

vaccine would be theoretically valuable and marketable.

Between 60-90% of HIV-infected patients in Africa without access to antiretrovirals experience bouts of diarrhea and EAEC strains have repeatedly been isolated from HIV-positive patients with diarrhea [17,144]. In one Central African Republic study, EAEC was isolated from 12.7% of 110 HIV-positive patients with diarrhea and none of 73 asymptomatic controls. EAEC was also strongly associated with persistent and bloody diarrhea [143]. In Senegal, EAEC were recovered from in 42 (27%) HIV-positive patients with diarrhea and was only rarely recovered in HIV-negative individuals [137]. Bernier *et al*. screened 25 EAEC isolates recovered from HIV-positive patients in Bangui, Central African Republic and Dakar, Senegal, for a number of virulence genes [143,144,195]. Unfortunately, only a small subset of strains from each study were investigated in this detail, making it difficult to link any of the detected genes to epidemiology or locale. As shown in a Nigerian study, adults are also susceptible to EAEC infection, irrespective of HIV-serostatus [14].

EAEC are commonly recovered from asymptomatic individuals. While this partly reflects strain heterogeneity, it may be related to infection load and also arises from inter-individual variation in susceptibility [18,196]. Thus individuals carrying EAEC strains may be reservoirs for bacteria that could cause disease in more susceptible individuals. Concerns have been raised by investigators in parts of Africa where asymptomatic carriage was very common about the possibility of asymptomatic carriers who are food handlers passing these strains to travellers, as well as of adults infecting young children [15,197]. In the United States, asymptomatic carriers at day care centers were show to facilitate EAEC transmission to susceptibles [198].

Non human reservoirs for EAEC have yet to be identified and little is known about risk factors for infection. An American study determined that EAEC can survive in low-pH sauces, and that eating salads, which often are consumed with low-pH condiments, has been identified as a potential risk factor in England [189, 199]. EAEC could potentially survive in low-pH fermented cereals fed to babies in many parts of Africa [200,201].

Worldwide, far too few investigators seek EAEC and a sensitive, specific and practical detection method is yet to be developed. It is becoming increasingly clear that while some EAEC strains are most certainly pathogens, others may not be, so that the category itself may require redefinition. In addition to bacterial heterogeneity, there are host variations in susceptibility to EAEC infection, at least some of which are heritable, making the definition of EAEC and the identification of risk factors difficult.

EAEC strains are emerging pathogens in the sense that humans have only recently become cognizant of them. For different reasons than EPEC, but with similar shortfalls, it becomes impossible to describe the early epidemiology of EAEC in Africa or to infer the impact that recent antidiarrheal interventions have had on EAEC epidemiology. All studies that have sought EAEC in Africa have found it to be one of the most common diarrheagenic categories, and most have highlighted the EAEC strains' associations with disease [14,15,18,20,55,66,134,136,143,197,202]. Far more studies have not sought EAEC and in those that have, methodology has varied widely. The gold standard diagnostic test is the HEp-2 adherence assay, in which EAEC strains form a characteristic "stacked brick" or "honeycomb" pattern as shown in Figure 2b. In order to perform the test, laboratories require facilities for tissue, as well as bacterial, culture. A skilled technician is needed to set up assays, and to prepare and read slides in what is a very cumbersome protocol. A DNA probe, CVD432, was developed as a diagnostic tool and is highly specific for EAEC strains carrying the somewhat variable aggregative adherence (pAA) virulence plasmid [203]. While the CVD432 probe has made it possible for more investigators to seek EAEC, it is insufficiently sensitive. Sensitivity in a range of studies has varied from below 20% to 89%, as compared to the gold standard HEp-2 adherence assay. The particularly low sensitivity in African studies is a reflection of the absence of strains from Africa in early studies evaluating the probe [203] (Table 2). There is, therefore, a need to make future diagnostic test evaluations more geographically inclusive and to seek adjuncts or alternatives to the CVD432 probe.

The requirement for tissue culture facilities and expertise does not permit many African laboratories to use the gold standard test for identifying EAEC. It was initially hoped that dispersin, an antiaggregative protein (Aap) secreted by many EAEC strains, could serve as a diagnostic target [204]. However, a recent report demonstrates that, like many other EAEC factors, Aap is produced by non-EAEC strains [205].

Table 2. Low sensitivity of the CVD 432 probe in Afican studies compared with the gold standard HEp-2 adherence assay.

Study location	CVD432 target detected by:	Patients with diarrhea	Association of aggregative adherent EAEC with diarrhea	Association of probe-positive EAEC with diarrhea	Sensitivity of CVD432 detection (versus Gold Standard HEp-2 adherence assay	Ref.
South West Nigeria	DNA hybridization	Children	p<0.04	NS	34/113	[63]
South West Nigeria	DNA hybridization	Adults	p=0.011	NS	14/24	[13]
Lambaréné, Gabon	PCR and DNA hybridization	Children	N/A	N/A	19/57	[130]
Central African Republic	PCR	Adults	NS	NS	0/14	[136]
Lwiro, Congo	DNA hybridization	Children	p<0.03	NS	44/79	[192]

Considerable effort has gone into increasing the sensitivity of DNA-based diagnostics for EAEC or devising alternate tests. Increasing the number of genetic loci sought can increase sensitivity of DNA-based methods for EAEC although most of the commonly sought targets are also plasmid-borne [18,55,206,207]. One phenotype initially anecdotally associated with EAEC in early studies is autoaggregation [184]. Subsequent research has identified at least four varieties of type IV pili, as well as non-structural adhesins that can account for this phenotype and it is likely that other autoadhesins exist. Albert *et al*. devised and evaluated an autoaggregation or clump-forming test for EAEC [208]. The original assessment used very few strains. In spite of efforts by Iwanaga *et al*. [209], who attached considerable finesse to interpretation, the clump test (although more sensitive than the CVD432 probe) is too non-specific for clinical diagnosis [210]. A quantitative biofilm test has also been proposed and has been evaluated in Tanzania [211,212] and may be a useful preliminary screen for EAEC; however, more definitive tests that can be performed in African laboratories are needed.

Many putative EAEC virulence factors are located on a partially conserved plasmid, pAA, which is marked by the CVD432 locus [191,192,213,214].Although pAA genes have been the focus of much study, they are only partially correlated with EAEC virulence or virulence-associated phenotypes [135,215,216]. Recent research is beginning to uncover chromosomal loci with promise as epidemiological and virulence markers [217-221].

The enteroaggregative heat stable toxin (EAST-1), encoded by the phage-borne *astA* gene, is present in pathogens other than EAEC and there have been varying results in studies that assessed its role in disease. In a Central African Republic study, although several EAEC virulence genes were detected, only *astA* was detected significantly more commonly in strains from patients with diarrhea than from controls ($P < 0.0001$)[68]. In Gabon, both *astA* and *pet*, encoding the EAEC plasmid-encoded toxin, were associated with disease [136]. However, a study performed in Nigeria, which screened for these genes and two other toxin-encoding loci—the Pic mucinase/ *Shigella* enterotoxin 1 genes (encoded in overlapping open-reading frames) and alpha-hemolysin—saw no association of any of them with pediatric diarrhea [135]. In that study, with the exception of aggregative adherence fimbriae II (AAF/II), no single factor was associated with disease, but strains bearing multiple virulence genes were more likely to be recovered from children with diarrhea, suggesting that EAEC virulence factors may work in concert or that the accumulation of multiple virulence factors may increase EAEC virulence [135].

The question as to whether there are EAEC lineages that are more virulent pathogens remains to be systematically addressed. There are important clues from two African studies of multiresistant EAEC strains. Sang *et al*. found that O44:H18 isolates were common in Kenya [222] and they have been reported as being significant globally. One O44:H18 strain, 042, has produced diarrhea in the greatest number of human volunteers. Recent

completion of the 042 genome offers the potential for comparative studies with other EAEC. As with EPEC, serotyping is helpful in roughly delineating EAEC lineages but multilocus sequence typing [126] shows more promise. Wallace-Gadsden *et al*. [223] identified one MLST sequence type complex, ST394, as an expanded clonal group in Nigeria. Interestingly, ST394 strains are closely related to 042, as well as to ST69, a globally disseminated antimicrobial resistant uropathogenic clonal group. Both brief reports suggest that there are hypervirulent EAEC lineages, which could be identified by MLST or other phylogenetic methods. These lineages are also multiply resistant. Antimicrobial resistance is a critical issue with EAEC strains, which appear to be more likely to harbor resistance genes than other *E. coli* isolated in the same area and may cause persistent diarrhea, for which antimicrobials are indicated [64,135,136,224]. Have these pathogens gained prominence as a result of selective pressure afforded by antimicrobials or because of an exceptional capacity to acquire mobile resistance elements?

Diffusely-adherent E. coli

As their title suggests, diffusely adherent *E. coli* (DAEC) show a diffuse pattern of adherence to epithelial cells (Figure 2c). As with EAEC, this definition does not identify a genetically homogenous group. Two DAEC strains did not produce diarrhea in a human challenge study; however, since the category is heterogeneous, no more strains have been tested, and failure to produce diarrhea in adults does not reveal lack of virulence in more vulnerable individuals, this does not rule out DAEC as enteropathogens [225,226]. In Maputo, Mozambique, HEp-2 adherent DAEC were identified in 125 (22.8%) patients and 42 (11%) controls ($P < 0.0001$) [20]. In that study, DAEC was more frequently detected than EPEC, ETEC, EIEC (identified by PCR) or EAEC (identified by HEp-2 adherence). However, while there is evidence from a few studies elsewhere associating these strains with disease, such an association has not been found in the few other African studies involving children [65] or HIV-positive patients [68].

It must be emphasized that too few investigators have sought DAEC at all and smaller and uncontrolled studies reveal that DAEC are widespread. They were identified in four HIV-positive patients with diarrhea in Kenya, in a study where they were as common as EAEC [17] and have also been reported from HIV-positive and HIV-negative patients with diarrhea in Tanzania [227]. In the Central African Republic, one study found DAEC in 8.2% of HIV-positive patients with diarrhea and only 1.4% of symptomless controls [143], again at a comparable rate with EAEC. Gassama-Sow *et al*. identified *afa* genes in about 190 (32%) of isolates recovered from HIV-positive and -negative patients, with and without diarrhea [137]. There was no association of *afa*-bearing strains with disease in any of those groups in that study. Some of the isolates recovered had virulence factors associated with other enterovirulent groups—EPEC (4), EAEC (14), EIEC (9)[137].

Only a subset of DAEC strains have Daa adhesins from the Afa/Dr family. Other diffuse adhesins include fimbrial and non-fimbrial adhesins AIDA-1, Sfa and P-pili [228], all of which are also found in uropathogenic *E. coli* (UPEC) and commensals. Their role in diarrheal disease, if any, is unclear. In one study, *afa*, *sfa* and *pap* were detected among DAEC isolates from Bangui but none of them, nor DAEC as a whole, were associated with chronic diarrhea in HIV-positive patients [68]. Recently, Guignot *et al*. have identified an autotransporter toxin, Sat, in UPEC and many diarrhea-associated DAEC [229]. Importantly, they showed that *sat* was significantly more commonly found in DAEC from children with diarrhea and UPEC than in DAEC from healthy children [229]. *sat* may represent a true DAEC virulence marker that future epidemiological studies, including those in Africa, may want to investigate. Several DAEC isolates obtained from Africa are negative for many known adhesins [143]. Such strains could show diffuse adherence and diarrheagenicity by other mechanisms. It must be mentioned that some diarrheagenic *E. coli* belonging to other categories produce diffuse adherence. This includes many ETEC strains bearing CFAs, as well as EHEC, STEC and atypical EPEC strains with accessory adhesins [230]. These strains are generally not categorized as DAEC.

Multiple existing probes recognize only a subset of DAEC strains and the commonly used DAEC probe, *daaC*, cross-reacts with some (but not all) EAEC [210,231]. Thus the association of DAEC in disease observed in some studies is equivocal [227] and even laboratories with molecular capability have faced difficulties conclusively identifying DAEC. Recently devised methods [210] may overcome these problems and more work is needed to define these more specifically and devise means for studying their

epidemiology. *daaD*-positive DAEC were, however, recently associated with disease in adults in Ghana in a study that employed definitive methodology [15].

Other potentially diarrheagenic E. coli *categories*

Cell detaching *E. coli* (CDEC), otherwise termed diarrhea-associated hemolytic *E. coli*, are hemolytic and detach cell monolayers from *in vitro* culture surfaces. The detaching activity is conferred by alpha-hemolysin and CDEC also possess pyelonephitis-associated pili and cytotoxic necrotizing factor toxin [232,233]. These virulence factors are found in *E. coli* isolates from invasive infections and a role in diarrheagenicity, if it exists, has yet to be established. However, Somali and Nigerian studies have reported CDEC strains from children with diarrhea. These studies observed that these strains belong to very few serotypes, commonly O4(:H40) and O1:H7, and are frequently lactose-negative [232,234-236]. They are also resistant to several antimicrobials by virtue of plasmid-borne resistance genes [232,234]. Some CDEC strains also bear aggregative adherence plasmids and in that sense are EAEC as well as CDEC, although the vast majority described from African locations lack aggregative adherence plasmids [143,232,233,237]. The significance of these findings remains unclear, particularly as very little is known about CDEC in general and strains that fit the CDEC profile commonly colonize asymptomatic people [238]. In addition to their recovery from children with diarrhea in Nigeria and Somalia, CDEC was associated with diarrhea in Australian aborigines and have been reported from other areas [136,237,239]. Many CDEC, particularly those reported from Africa, are lactose negative and would be missed in studies that use lactose fermentation as a preliminary screen for diarrheagenic *E. coli*.

Cytolethal distending toxins cause distension by inhibiting cyclomodulins (and thereby stalling the cell cycle). At least five different cytolethal distending toxins have been described in *E. coli*, and type I is phage borne [240-242]. Only two African studies have sought cytolethal distending toxin-producing *E. coli* (CLDTEC). Okeke *et al*. [65] identified only three CLDTEC from Nigeria and they showed no association with disease. More recently, Mendez-Arancibia *et al*. found that 12.8% of 86 EAEC strains from Tanzania were cytolethal distending toxin-encoding gene-positive [212]. Epidemiological studies from elsewhere have detected only a low frequency of CLDTEC and an association of this toxin with EPEC or EHEC-linked serovars. The role of this toxin in human disease remains to be established and rather than a distinct category, CLDTEC may represent other pathotypes that are converted by lysogenization with a *cdt*-bearing lambdoid phage [242].

Diagnosis of diarrheagenic E. coli*: what is it needed and how can it be implemented?*

All known categories of diarrheagenic *E. coli* have been detected in Africa. Given the unmeasured but significant contribution that these pathogens make to the burden of illness, the epidemiology of diarrheagenic *E. coli* in Africa is poorly understood, and very unevenly studied. Half of published studies abstracted on Medline focus on *E. coli* from just three countries: Kenya, Nigeria and South Africa. Even within these countries, studies have been restricted to a handful of locales and at-risk populations. Medline now abstracts some prominent African journals but for one-third of the nations in sub-Saharan Africa, there are no citations relevant to diarrheagenic *E. coli*. The dearth of information is reflective of the lack of capacity to detect diarrheagenic *E. coli* for research or clinical purposes, even though they are among the most common causes of disease. Multi-country studies have rarely included an African site and when they do, diarrheagenic *E. coli* are often not included in the range of target organisms [243,244]. In many recent epidemiology studies that have been performed in Africa, culture has been done at the point of collection while delineation of diarrheagenic pathotypes has occurred overseas, usually in Europe or North America [67,245]. While this has provided otherwise unobtainable data, it has served endemic areas less well than would have local detection and outbreaks are frequently detected several months or years after they occurred [14,65,171].

As a surrogate for etiologic data from residents in endemic areas, travellers sampled on site or, more commonly, upon return have been used to estimate the prevalence of enteric pathogens in different parts of Africa. This too has provided much useful data in that etiologic agents of travellers' and infantile diarrhea are often similar; however, they are occasionally not and until any region is well-studied, the degree of overlap remains a subject of conjecture [3]. Also, many travellers do not share risk factors with endemic residents [96]. Most of the published data on diarrheagenic *E. coli* is from studies where infected people or pathogenic strains were exported. These studies are best viewed as pilots for more

informative surveys that must be executed in the near future.

A recently initiated Global Enterics Multi-Center study (GEMS) at eight sites in developing countries (*Centro de Investigaçao em Saude da Manhiça Manhiça*, Mozambique; Medical Research Council, Basse, Gambia; CDC/Kenya Medical Research Institute Research Station, Kisumu, Kenya; *Centre pour le Développement des Vaccins du Mali* (CVD-Mali), Bamako, Mali, to name those in Africa) should go a long way towards closing the knowledge-gap for diarrheagenic *E. coli* and other enteric pathogens [246]. Smaller research initiatives at non-participating sites will hopefully provide a more complete picture that covers much of the variable pathogen terrain south of the Sahara. Where possible, future studies need to use tests that are sensitive and specific for multiple diarrheagenic *E. coli* pathotypes, without bias towards subtypes that have epidemiological relevance in other parts of the world. As such methods necessarily require molecular biology and/or tissue culture, there is need to increase capacity for both on the continent. While there have been fewer studies using appropriate diagnostic methodology, and these studies have typically been very small, the wealth of information they have generated argues for more widespread use of appropriate diagnostic tests.

It is unrealistic, and indeed unnecessary, for every diarrheal specimen in Africa to be screened for diarrheagenic *E. coli*. Most diarrheagenic *E. coli* infections can, and do resolve spontaneously. Furthermore, stool culture is expensive and any evaluation that includes diarrheagenic *E. coli* costs much more, although the increase in diagnostic yield could increase the cost effectiveness of exhaustive investigations. Nonetheless, the cost and time required to diagnose laboratory-evaluated stools often does not justify routine diagnosis [247]. For clinical purposes, it would be ideal if bloody, persistent, hospital-acquired or suspected outbreaks are investigated for diarrheagenic *E. coli* at sentinel sites. On the research side, epidemiological surveys are needed to inform empiric treatment guidelines and vaccine development. All these functions could be performed at reference laboratories equipped to detect diarrheagenic *E. coli*. Considering the important contribution these strains make to infant mortality and growth and developmental mortality, laboratories with this capacity may be needed more in many parts of Africa than elsewhere.

Bacterial isolation protocols of many studies may exclude important pathogens. Screening multiple strains per specimen increases the chance of detecting pathogenic strains but many investigators are unable to do this because of resource limitations, in which case it may be beneficial to screen DNA isolated from stool [248-250]. Most studies seeking diarrheagenic *E. coli* alone select only lactose-positive colonies, which underestimates some pathotypes, notably EIEC and CDEC [232,236]. As with epidemiology, the molecular pathogenesis of many pathogens that are more prevalent in Africa than elsewhere is poorly understood and African laboratories rarely undertake in-depth characterization of locally isolated pathogens. Isolate archival is a challenge for many labs; transfer to other locations is difficult; and African scientists need to play a more direct role in molecular studies of principal local pathogens [251,252].

Clinically relevant unanswered questions about the biology of diarrheagenic E. coli

Asymptomatic carriage: Healthy carriage of enteric pathogens in general and diarrheagenic *E. coli* in particular is high in many African studies [15,65,132]. Uncontrolled studies are convenient and use fewer resources but their cost effectiveness must be questioned when carriage rates are so high. In one Tanzanian study, 10% of children with diarrhea were infected with ETEC, but this pathogen was detected in as many as 5% of controls [253] and in another study from the same region, carriage rates were so high that no diarrheagenic *E. coli* category was associated with disease [132]. Carriage rates are often even higher for EAEC and DAEC, pointing to inter-strain variability and the likelihood that some strains are not pathogens, or at least less virulent than others. High carriage rates mean that the epidemiology of these pathogens can best be understood by in-depth characterization of isolates from large controlled studies. However, due to resource limitations, most African studies, even when controlled, have been very small, short, and localized. Larger studies are more likely to uncover sub-type associations with disease when these exist and to identify micro-outbreaks. Furthermore, rarely performed population-based studies are the only way to link isolation rates to disease burden.

Mixed infections: Available evidence shows that mixed infections are very common in parts of Africa [14,65,94,254]. They include infections with diarrheagenic *E. coli* and other pathogens as well as

multiple categories of diarrheagenic *E. coli* and multiple strains of a single category. Interactions between different pathogens have not been commonly studied but two reports suggest that mixed infections that include diarrheagenic *E. coli* may be exceptionally deleterious [255,256].

Reservoirs and transmission: Certain weaning foods and other environmental factors may confer protection from infection [201]. There are also host factors in carriage as older individuals are even more likely to carry pathogens asymptomatically and hyper-susceptibility is heritable [15,196]. Understanding host resistance to diarrheal disease could assist in devising protective strategies for the more vulnerable. Pathogenic bacteria are known to multiply in infant foods and have been recovered from infant feeding implements as well as from adult care-givers, predisposing young children to infection [67,257-259]. Contaminated water and person-to-person transfer of these organisms are probably predominant routes for spread. Water supplies, sanitation and frequency of hand-washing with soap could be improved in most parts of Africa [260] and these interventions are proven to prevent the acquisition and spread of diarrheagenic *E. coli* and other pathogens [129,140,261-264].

Bovines, such as Zebus, have been implicated as reservoirs for EHEC, and contaminated water must make the largest contribution to diarrheal prevalence, but other reservoirs for diarrheagenic *E. coli* are not known. One study has found *Shigella* in salads, and EAEC and DAEC are associated with tomato sauce, and starch foods prepared or served with this sauce, in Accra, Ghana, possibly from contaminated food but likely not unrelated to food service [140]. Data are lacking from elsewhere. Close contact of many Africans with animals and evidence of zoonotic transmission of enteric organisms means that pathotypes with an animal reservoir will have a high chance of being communicated to humans [265].

Antimicrobial resistance: Most diarrheas are self-limiting and the principal treatment is rehydration until the patient's immune system clears the infection. In some diarrheas, notably those caused by Shiga-toxin producing *E. coli*, antimicrobials may be contra-indicated as they release more toxins from lysed or SOS-induced bacteria. Conversely, antimicrobials reduce shedding of *Vibrio cholerae* and *Sh. dystenteriae* thereby helping to contain epidemics. When pathogens are invasive, antimicrobials may be essential to clear them and prevent long-term sequelae, and for persistent infections, antimicrobial agents may be the only recourse. Diarrheagenic *E. coli* span the spectrum of these examples. EAEC and EPEC are commonly associated with persistent diarrhea, EIEC and *Shigella* cause invasive dysentery, while EHEC and STEC produce Shiga toxins. Scant knowledge of the role of more recently described diarrheagenic *E. coli* pathotypes means that, in the cases where antimicrobials are required, treatment protocols may not be optimal. For example, bloody diarrhea is presumed to be due to *Shigella* or *Entamoeba* infection in most parts of Africa and is therefore treated with antimicrobials, which are contra-indicated in Shiga-toxin-producing *E. coli*.

In the majority of diarrheas for which antimicrobials are not indicated, they are often prescribed because of the difficulty in distinguishing self-resolving infections from drug-indicated ones. Prescribers and self-medicators would use antimicrobials less in this way if there was a way to determine which infections might be life-threatening. The present situation typified by inexpensive drugs and costly, difficult-to-access diagnostic tests make it unlikely that antimicrobial misuse can be reasonably curtailed in many places [27,266]. Even when antimicrobials are indicated, more expensive antimicrobials may be needlessly prescribed because susceptibility testing, or local surveillance data, is unavailable [267].

When antimicrobials are required, more recent studies have shown that common *E. coli* pathotypes such as EPEC, ETEC, *Shigella*, EIEC, and EAEC are resistant to almost all drugs available and affordable to patients in this part of the world so that optimal treatments do not exist [134,268]. Results from a Tanzanian study showed that 25-90% of ETEC, EAEC and EPEC isolates were resistant to ampicillin, trimethoprim-sulphamethoxazole, tetracycline and chloramphenicol [64]. Resistance to the quinolones, although then uncommon, was detected. Over 80% of pathogens belonging to all three classes were resistant to ampicillin and trimethoprim-sulphamethoxazole, which represent the only options for treatment of pediatric persistent or invasive diarrheas in many areas [64]. Comparable results have been seen in bacterial isolates from other studies including HIV-positive patients with diarrhea in Senegal and from EAEC from Nigerian children where strains from children with diarrhea were more likely to show resistance to commonly used agents than strains from healthy children [56,135,136,144].

It is important to read these findings in context. Resistance rates in many African countries, although very high, have been lower than in other parts of the developing world. In one comparative study of travellers' diarrhea bacterial isolates, strains from Mombasa, Kenya, were significantly less likely to be resistant to three or more agents than isolates from Goa, India, and Montego Bay Jamaica [96]. However, resistance in the Mombasa isolates was, at 30%, high enough to warrant concern. In particular, resistance rates to trimethoprim, sulphonamides, tetracyclines, gentamicin and streptomycin were comparable or greater in Mombasa than isolates from other areas.

Case fatality rates in outbreaks caused by antimicrobial resistant *Shigella* and other diarrheal pathogens have been raised by resistance and, in these situations, susceptibility testing performed early in the epidemic could reduce mortality [173,269]. Due to resistance, quinolones have replaced doxycycline and trimethoprim-sulphamethoxazole as drugs of choice for travellers diarrhea, but quinolone resistance has since emerged and is increasing. The only promising alternative is rifaximin, a recently licensed, non-absorbable antimicrobial that can be used to treat infections by non-invasive *E. coli* pathotypes [85,119,270-273]. Availability and costs mean that rifamixin is presently limited to adjusting recommendations for travellers from affluent countries to compensate for the emergence of resistance, a niche occupied until now by ciprofloxacin. Travellers with access to next-generation antimicrobials bring with them selective pressure for Africa's reserve medicines and drugs of the future.

Over the last 20 years antimicrobial resistance has been reported for all classes of diarrheagenic *E. coli* and specifically from African isolates [64,274]. African countries must prioritize resistance containment if the effectiveness of affordable drugs is to be maintained. Non-antibiotic strategies for diarrhea disease management would conserve antimicrobials for those cases where there are no alternatives and would reduce the overall selective pressure from these drugs. Nutritional supplementation, in particular Zinc, has been proposed as a means for promoting small-bowel repair after infection, leading to shorter episodes of persistent diarrhea and resistance to reinfection [275]. Some of these strategies could reduce the burden of disease from diarrheagenic *E. coli*. Preventing diarrheal episodes in the first place must be a primary goal.

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